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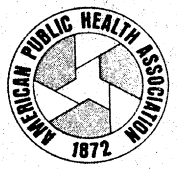
Standard Methods for the Examination of Dairy Products



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CHAPTER 4

CULTURE MEDIA AND PREPARATION

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4.1 Introduction

Accuracy and precision of results obtained from microbiological testing of dairy products are dependent to a large extent on culture media employed and care exerted by technologists in their use. Therefore, it is important that media always be prepared and handled properly.

The following culture media are those mentioned in other chapters of this book; names given them are for the most part those in the literature. In most instances media listed here can be obtained in dehydrated form, and it is strongly recommended that such media be employed. Directions for making them are found on the bottle labels and in manufacturer's technical bulletins. However, two of the media listed below* may be unavailable commercially. Section 4.9 of this chapter provides the necessary information for preparation of these media.

A K Medium #2 [2]
Antibiotic Medium #1
Brilliant Green Lactose Bile Broth, 2%
Casein Soy Peptone Agar
Casein Soy Peptone Agar with Polysorbate (Tween) 80 and Lecithin
*Citrate Azide Agar [15, 17] [See 4.9][1]
Eosin Methylene Blue Agar, Levine [10]
Lactose Broth
MF-Endo Broth [6]
Mueller-Hinton Agar [13]
Nutrient Agar
Nutrient Broth
Potato Glucose Agar (acidified)
*Rose Bengal Agar with Chlortetracycline [5]
Standard Methods Agar:
  Pancreatic digest of casein

ISC Liaison: W.J. Hausler, Jr.

Yeast extract
Glucose
Agar
Standard Methods Agar with Polysorbate (Tween) 80 and Lecithin [3]
Violet Red Bile Agar

A. Dehydrated media:

Dehydrated media should be stored in sealed containers in a cool, dry place and protected from light. If the environment is hot and humid, media may be stored in a refrigerator or freezer, as preferred. Properly stored, most media should be stable for at least three years; however, purchases should be planned to permit a complete turnover within a year or two.

After the first usage, when the seal has been broken, quality of the medium may depend upon the storage environment. It is recommended that a suitable package size be purchased to minimize repeated use of material from an unsealed container.

The unsealed container may admit air and moisture which can initiate reactions that result in reduced productivity of the medium. Some chemical or microbiological contamination may also take place if unclean spatulas are used to transfer the material.

B. Prepared media:

The "life" of any prepared medium, whether in tubes, bottles, or plates, depends on the condition of storage and the type of medium. Prepared media should not be stored unless protected against water loss. This may be accomplished by using screw-capped tubes and bottles instead of cotton-plugged containers. Prepared plates should be stored in moisture-proof containers, such as plastic bags, to minimize moisture loss. Because of the instability of prepared culture media, it is advisable to use prepared plates no more than one week old and media in screw-capped tubes no more than six months old. If dehydration has occurred, media should be discarded. All prepared media whether in plates or tubes, should be stored between 2–8 C.

4.2 Basic Steps in Medium Preparation

A. Weigh carefully the proper amount of the dehydrated base medium or the correct proportion of constituent ingredients.

B. Place the requisite amount of microbiologically suitable (distilled, deionized or otherwise suitably treated) water into a suitable container (e.g., borosilicate glass or stainless steel).

C. Add the weighed material(s) to part of the water. Mix with a stirring rod. Add remaining water and mix again.

D. Heat, if necessary to effect complete solution, by boiling on an asbestos-centered wire gauze over a free flame or over an electric hot plate, agitating frequently to prevent burning of medium at the bottom of the container. A non-pressurized free-flowing steam unit may also be used. Media

containing agar should be boiled to insure solution of the agar. Prolonged boiling may cause undesirable foaming; this can be reduced by holding the flask in cold water for a few seconds after initial boiling has been accomplished. Restore water, if necessary, to compensate for loss by evaporation. An alternative method of preparing agar is to add the dry ingredients to the requisite amount of water in the flask, mix to disperse lumps, and autoclave for the required time. After autoclaving, leave the flask in the autoclave with the door open for 5 min, then place in a water bath and carefully agitate until any superheated steam has escaped. Then mix vigorously. Care must be taken to avoid agitating the freshly autoclaved agar as the superheated solution may boil violently.

E. Determine pH of the medium and adjust if necessary. The pH of agar media is best determined before boiling.

F. Distribute medium into suitable containers. This can be most easily accomplished for tubed liquid media by using automatic pipettors commercially available through laboratory supply houses. A handheld adjustable pipettor is most suitable for small numbers of test tubes, whereas an electrically operated machine is more satisfactory for larger numbers. In each instance, before use, machines must be flushed with microbiologically suitable water, followed by medium until the water has been completely removed from the system. After use, the apparatus must be flushed with warm water, detergent solution, tap water, and distilled water. If any residues accumulate the apparatus should be disassembled and scrubbed. A small amount of water should be left in the syringe portion to prevent drying out and subsequent "freezing" of the plunger and barrel.

G. The amount of medium per container should be limited so that no point within the volume of medium is more than 2.5 cm from the top surface of medium or area of medium interfacing with the container (to insure rapid equilibration of temperature when placed in a waterbath).

H. Sterilize at 121 C (149.8 F) for 15 min or according to recommended procedures for each medium. Rechecking of pH before use of medium is recommended [4.3].

I. Autoclaving efficiency is greatly reduced by overloading the autoclave or by improper spacing of containers. For good results, separate containers by at least one-half inch in all directions and do not use volumes of media in excess of 3–4% of the volume of the autoclave. If larger volumes are used, sterilizing times should be increased [see 4.4(A)].

J. To use, melt and hold solid media at 44–46 C until used, but not exceeding 24 hours. If a precipitate forms, discard media. Do not re-melt media.

NOTE 1. Chemicals and substrates such as carbohydrates must be of reagent grade unless otherwise specified. Follow manufacturer's instructions for storing stock reagents. Discontinue use of chemicals showing any evidence (color change, for example) of contamination, decomposition, or hydration.

NOTE 2. Automatic agar makers with a complete sterilization cycle are on the market and they may be of value to some laboratories.

4.3 Adjustment of Reaction (pH)

Determine the hydrogen-ion concentration (pH) of culture media at 25 C (77 F) electrometrically. This temperature is used in commercial production of media and should be used in the laboratory to determine the pH of media before use. Determinations made at 45 C (113 F) to take advantage of the fluid state of agar are not accurate, differing significantly from those obtained at 25 C.[21]

A. Electrometric procedure (potentiometer):

Allow electrodes of the instrument to equilibrate (some models may require 30 min) to the temperature at which the determination is to be made, and adjust the buffer solution to the same temperature before testing the instrument. Only buffers referenced to the National Bureau of Standards are recommended. The pH of the buffer solution should be in the range of the pH of the medium to be tested. The temperature of test solution and buffer should be the same; room temperature (25 C) is generally used for convenience. For solid media, macerate a suitable aliquot thoroughly with a glass rod before inserting the electrodes. Be sure that the temperature is maintained until the reading is complete. If in doubt, repeat the determination. Do not dilute test solutions or buffers.

NOTE: Plating media are not highly buffered and this can cause confusion. Meters are designed only to show a difference in pH between two solutions at the same temperature. Completely anomalous observations will be obtained if the meter is used under other conditions. Temperature compensators of pH meters do not permit correction of a temperature difference between test solution and reference buffer. The reason for this is that the H^+ concentration of the buffer changes with temperature. Temperature compensators should be set at the temperature at which measurement is made. Since the emf/pH ratio is less at 25 C than at 45 C, for example, a correction factor must be used; this is built into the instrument and allows one to determine the correct pH directly.

4.4 Sterilization and Storage

Before sterilization, bring the medium to boiling temperature, stirring frequently. Restore lost water if necessary [4.2(D)] and autoclave. Because the pH of a medium may change during sterilization and because of possible browning reactions, it is important not to exceed the recommended temperature and time. Reduce pressure with reasonable promptness (but no less than 15 min) to prevent undue changes in the nutritional properties of the medium and remove from the autoclave when atmospheric pressure is obtained. Preferably use flasks or bottles from which melted medium may be

poured into plates. Optionally use test tubes containing 15–20-ml amounts for pouring melted medium into plates.

Prepare medium in quantities so that, if stored, it will be used before loss of moisture through evaporation becomes evident. To prevent contamination and excessive evaporation of moisture from a medium in flasks and bottles during storage, optionally fit pliable aluminum foil, rubber, plastic, parchment or heavy kraft paper, or viscose caps securely over closures before autoclaving. Use of screw-cap or crown (cork-and-seal type) closures on containers appreciably reduces such risks. If tubed media are used within a short time, commercially available polypropylene or stainless steel closures may be used. Media should be stored at 2–8 C in a dry dust-free area and should not be exposed to direct sunlight.

A. Steam sterilization:

Steam-sterilize media, water and materials (such as rubber, cork, cotton, paper, heat-stable plastic tubes and closures) that are likely to be charred in the dry-air sterilizer by autoclaving at 121 C for not less than 15 minutes. Autoclave media and dilution blanks within an hour of preparation. Slightly loosen stoppers to allow passage of steam into and air from closed containers when autoclaved. Place one or more spore controls in the center of the load, preferably within a container similar to those being processed. Make certain that the load is loosely packed [4.2]. Before allowing steam pressure to rise, automatically or manually expel all air from the sterilizer through an exhaust valve of suitable size. If manual means are used, make sure free steam is being exhausted before pressurization begins. Because temperature obtained at a constant pressure of saturated-steam will vary according to atmospheric pressure, rely only on a properly operating and calibrated thermometer rather than a pressure gauge to insure sterilization.

Avoid overloading autoclaves so that the rate of air exhaust or heating is not appreciably delayed [4.2]. The autoclave should reach 121 C slowly but within 20 min after starting the air-exhaust operation. Contrary to popular belief, a rapid come-up time in an autoclave does not necessarily result in more efficient autoclaving. This results from failure of steam to replace air when steam enters the autoclave too quickly. A steam flow which is too slow also results in decreased efficiency because air-steam mixtures are forming.

Where non-liquid materials with slow heat conductance are to be sterilized, or where the packing arrangement or volume of materials otherwise retards penetration of heat, allow extra time for materials to reach 121 C before beginning to time the sterilization period and, if necessary, use longer sterilization periods to insure sterility.

After sterilization, *gradually* reduce pressure within the autoclave (no less than 15 min is recommended) since liquids may be at temperatures above their boiling point at atmospheric pressure. This is necessary because liquids can be lost through boiling when lowering the pressure too rapidly. When

sterilizing dry materials, such as sampling equipment or empty sample bottles, pressure may be released rapidly at the end of the 15-min holding period at 121 C. This prevents collection of condensate and speeds up drying of paper-wrapped equipment.

Decontamination of used plates, pipets, tubes, etc. should be done routinely in all microbiological laboratories. Dairy products, especially raw milk, may harbor potential pathogens such as *Staphylococcus*, *Streptococcus*, etc. The same principles apply for loading the sterilizer and sterilization as for media preparation. Plastic petri dishes are most conveniently sterilized by placing them in heat-resistant autoclaveable bags. When large numbers of petri dishes are autoclaved, sterilization time should be at least 30 minutes. Insufficient sterilization of plastic petri dishes is indicated when they retain some degree of their original shape; properly autoclaved plates become amorphous. This property by itself, however, is not an indication of sterility.

B. Hot-air sterilization:

Sterilize equipment with dry heat in hot-air sterilizers so that materials at the center of the load are heated to not less than 170 C (338 F) for not less than 1 hour (this usually requires exposure for about 2 hr at 170 C). To insure sterility, do not crowd the oven, and when the oven is loaded to capacity, preferably use a longer period or slightly higher temperature. Spore packs or thermocouples should be used in doubtful situations.

4.5 Quality Control

This chapter on preparation of culture media is in itself a discussion of day-to-day quality control practices; however, specific mention should be made concerning the advisability of routine adherance to simple quality control measures involved with preparation of culture media for use in standardized procedures.

The following items represent minimal but adequate quality control procedures that may be used by laboratories using standard methods to analyze dairy products. Many of them are mentioned elsewhere in this chapter, but are summarized here to emphasize their contribution to quality control in media production. A portion of the items was taken from a DHEW publication.[12]

A. Date the label of each bottle of dehydrated medium indicating when it was received and when it was first opened.

B. Store dehydrated media in tightly capped bottles in a cool, dry place protected from light, or in a refrigerator if necessary. Keep no more than a 6-month to a year's supply on hand, being sure to use older stocks first. Dehydrated media should be free flowing powders; if a change is noted in this property, or in color, discard.

C. Whenever possible, commercial dehydrated media should be used.

D. Complete mixing of medium to form a homogeneous solution in water

is necessary before sterilization and dispensing. Stirring which causes foaming should be avoided.

E. Performance of autoclave should be monitored by either a continuous temperature recording device in combination with properly placed indicator strips or discs, or spore strips or suspensions. The record for each "run" should be dated, numbered, and filed.

F. Limit heating of the medium to the minimum necessary to insure solution and sterilization. When the autoclave has reached atmospheric pressure, it should be opened immediately following the recommended cycle. Prolonged storage in a water bath should be avoided.

G. Check the final pH of each lot of medium, which should be cooled to room temperature. The pH of agar is obtained using a slurry of the medium in a beaker.

H. Aseptic technique should be followed strictly during dispensing of sterilized material. Hands should not touch any part of the dispensing tubing which comes in contact with sterile material. During interruptions in a dispensing cycle, the spout of a dispenser train should be placed in a sterile glass container. A dispensing cycle should not be interrupted except in an emergency.

I. Moving parts of any dispensing apparatus should be oiled or greased as indicated at least once a month. Any leaks should be corrected immediately. Accuracy of dispensation should be checked with a graduated container at the beginning of each dispensing cycle.

J. Media containing dyes should be protected from light by storage in a dark room, in a dark glass bottle, or by wrapping with brown paper.

K. Each container of autoclaved medium should be labeled with the name of the medium and the autoclave "run number."

L. Inclusive dates during which each lot and container of dehydrated medium was used should be recorded for possible future troubleshooting.

M. Each lot should be inspected visually before use for volume, tightness of closures, clarity, color, consistency, and completeness of label.

4.6 Suitability of Water for Microbiological Applications

Only water that has been treated to free it from traces of dissolved metals and bactericidal and inhibitory compounds should be used to prepare culture media, reagents, and dilution blanks. However, for routine use in dairy product analysis it is the growth medium or dilution system prepared with the water which is of concern, not the water itself. Culture media provide considerable protection from toxic agents present in water;[16] therefore, the primary interest in water quality for routine applications is in its use as a dilution fluid. A procedure to determine if dilution of a sample may have a toxic effect is given in Section 4.7(C).

However, if special circumstances arise which suggest that water *per se* be examined, specific resistance in ohms may be determined as a rough indication of distilled water quality, if the apparatus is available. Generally,

between 400,000 and 500,000 ohms specific resistance is the breakpoint between acceptable and unacceptable distilled water. A procedure to determine specific resistance is given in a publication of the American Society of Medical Technologists (ASMT) entitled *Reagent Grade Water*.[22] However, only ionic concentration is measured by specific resistance; presence of organic substances is not detected by this procedure. If organic contamination is suspected an experienced chemist should be contacted to make the determination.

Distillate from chlorinated water occasionally contains significant amounts of free chlorine, even when passed through an ion-exchange resin column before use. If distilled or treated water gives color with an orthotolidine test,[19] it should be neutralized with sodium thiosulfate before use for milk dilution blanks.

4.7 Preparation of Phosphate-buffered Dilution Water and Testing for Toxicity

A. Stock phosphate buffer and magnesium sulfate solutions:

1. Phosphate buffer: To prepare stock solution, preferably use previously adjusted dehydrated buffer. Alternatively, dissolve 34 g of potassium dihydrogen phosphate (KH_2PO_4) in 500 ml of distilled water, adjust to pH 7.2 with 1 *N* NaOH solution, and make up to 1 liter with distilled water. If desired, place in small vials, sterilize at 121 C for 15 min, and store in refrigerator to prevent microbial growth in the buffer before use.

2. Magnesium sulfate: To prepare stock solution, measure 50 g of $MgSO_4 \cdot 7H_2O$ into a 1-liter volumetric flask and add distilled water to make 1 liter of solution.

B. Buffered dilution water:

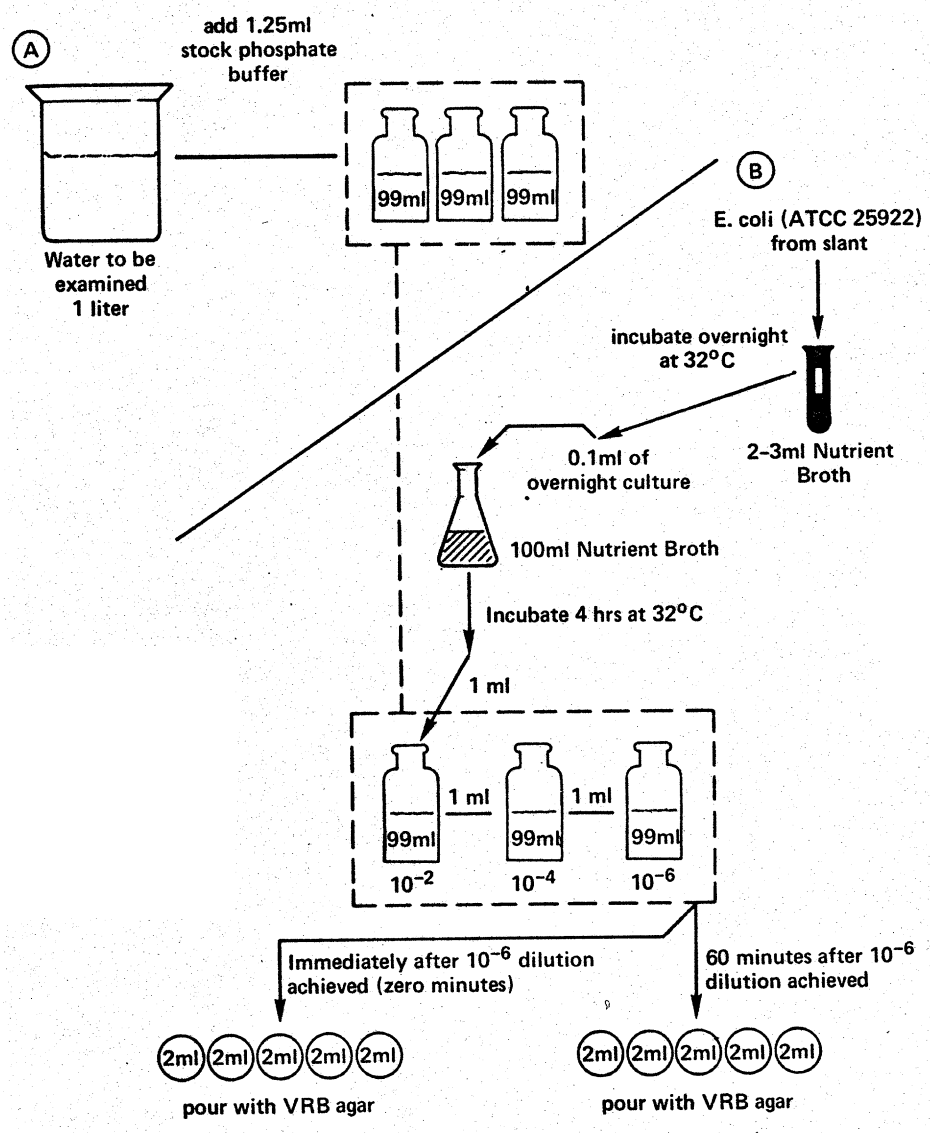
To prepare dilution water, add 1.25 ml of stock phosphate buffer solution and 5 ml of stock $MgSO_4 \cdot 7H_2O$ solution to distilled water and make up to 1 liter. Dispense as desired and autoclave at 121 C for 15 minutes. Addition of magnesium sulfate improves recovery of organisms with metabolic injury which may be induced by toxic properties in the dilution water.[7, 11]

C. Interval Plating Procedure:

Some data [4] indicate existence of toxic or inhibitory materials in unbuffered treated water when used as a diluent. When treated water is used as a base for buffered diluents (and also when tap water must be used, which is contrary to good laboratory practice because of variable composition and progressive precipitation of mineral salts onto walls of dilution bottles), a periodic check should be made for presence of toxic substances (such as chlorine, copper, ammonium, etc.).

To determine any toxicity in prepared phosphate buffered dilution water, the Interval Plating Procedure [8] as described herein should be done rou-

tinely (at least weekly) along with the normal workload. The procedure, shown schematically in Figure 4:1, has not been subjected to collaborative testing, but comparative studies have been made which indicate that the test sufficiently sensitive and satisfactory for use in laboratories that routinely



Incubate plates overnight at 32°C. Count.
Compute average of five plates for each time.
Compute % change between zero and 60 minutes.

Figure 4:1. Interval Plating Procedure

test milk. Because the test is easy to do and because it is the most workable system available, laboratory workers are urged to use this procedure.

The Interval Plating Procedure is a check on the dilution system as a whole, not just the treated water which was used to prepare it. Detergent residues which have persisted after washing bottles, phenolic or other residues in new caps, or chemical contaminants in reagents used to prepare stock solutions may lead to a toxic dilution system, when treated water that was used may be completely acceptable.

1. Procedure:

a. The treated water to be examined is made into dilution water by the procedure described in 4.7(B). This is then dispensed in 99-ml amounts into standard dilution bottles and autoclaved at 121 C for 15 minutes.

b. *Escherichia coli* (ATCC 25922) is used as the test organism. On the day before the Interval Plating Procedure is to be done, 2–3 ml of Nutrient Broth is inoculated from a nutrient agar slant culture and incubated at 32 C for 18 hours. One-tenth milliliter of the broth culture is then added to 100 ml of Nutrient Broth in a flask and incubated for 4 hr at 32 C to obtain a culture in the mid-log phase of growth. The 4-hr culture is then siluted serially to 10^{-6} in three of the dilution blanks prepared as indicated in a. The 10^{-6} dilution is immediately plated as the "zero" time dilution by measuring 2 ml of the dilution into each of five plates and mixing with Violet Red Bile Agar maintained in a water bath at 45 C. The culture is allowed to remain in the dilution water at room temperature for 60 min, and then plated again using the same protocol as for the "zero" time plating. The plates are then incubated at 32 C for 18–24 hours. Colonies are enumerated and the following calculation is made:

$$\frac{\text{Mean Colony Count (60 min)—Mean Colony Count (0 min)}}{\text{Mean Colony Count (0 min)}} \times 100$$

= % change in population between 0 and 60 minutes

The per cent change in colony count between zero and 60 min should not exceed ± 15% to be considered acceptable.

4.8 Cleaning Glassware and Testing for Detergent Residues

Modern detergents are very effective for cleaning laboratory glassware. Most of these are of the anionic type, usually with alkaline builders such as phosphates, carbonates, or silicates. Some detergents, especially the cationic type (quaternary ammonium compounds or "quats"), are highly bactericidal and great care must be exercised to ensure their removal. Detergents and soaps have a great affinity for all surfaces and because of this characteristic they displace dirt and allow it to be easily washed away. However, because of this same characteristic, they are difficult to remove completely.

A. Cleaning glassware:

Most common detergents in laboratory use are satisfactory for general purposes; however, occasionally deposits of "milk stone" or calcium salts are encountered which resist cleaning by ordinary means. These salts must be removed by rinsing glassware several minutes in acid solutions before effective cleansing can be achieved. A suitable solution for removing milkstone contains dichromate and sulfuric acid. This is prepared by dissolving 40 g of finely ground potassium dichromate in 150 ml of treated water. Place in a pyrex vessel and add very slowly with continuous stirring 330 ml of sulfuric acid such as used in the Babcock test for milkfat. During addition of acid the vessel containing the dichromate solution should be cooled by placing it in a cold water bath such as a stoppered sink. The laboratory worker should wear eye protection when making the cleaning solution.

The detergent wash is best done with hot water after preliminary rinsing with warm water to remove most of the dirt. Soaking aids in removal of stubborn residues. Glassware having residues which are not removed by the detergent action should be immersed for 24 hr in the acid-dichromate cleaning solution and then rinsed thoroughly in tap and distilled water. Traces of acid on glassware can be detected with pH indicator paper after the last rinse. Six to 12 rinses with running tap water followed by several rinses with distilled water are necessary for complete removal of detergent.

B. Detergent residues:

If doubt remains as to the effectiveness of rinsing, especially if a quaternary ammonium compound has been used, the following procedure may be used to detect bactericidal residues: glass petri dishes are prepared in three ways: one set of three is washed and sterilized by the usual method; a second set of three is washed in the acid cleaning solution, then in castile soap. They are then rinsed four times in tap water and six times in distilled water before sterilizing. A third set of petri dishes is dipped in the presently-used detergent solution and sterilized without rinsing.

A sample of milk is plated in triplicate in these dishes and colonies are counted after two days at 32 C. There should be no significant difference in counts between the first and second sets of plates. A reduction in bacterial count or diminished size of colonies may be apparent in plates of group three if the detergent in use is bactericidal or bacteriostatic.

4.9 Formulas of Culture Media and Directions to Prepare Media for Use

This section contains formulas and preparation directions for two culture media mentioned in this publication which may not be commercially available. The formula and specifications for Standard Methods Agar have been retained since *Standard Methods for the Examination of Dairy Products* is the original reference for this medium. In addition, comments on preparation and use of a few of the commercially prepared media are included.

A. Citrate Azide Agar:[15, 17]

Yeast extract	10	g
Casitone or trypticase (USP pancreatic digest of casein)	10	g
Sodium citrate	20	g
Sodium azide	0.4	g
Ditetrazolium chloride (tetrazolium blue)	0.01	g
Agar	15	g
Microbiologically suitable water to make	1	liter

To prepare the basic medium, do not add sodium azide or tetrazolium blue. Heat to boiling to dissolve other ingredients. Dispense in 100-ml amounts and autoclave at 121 C for 20 minutes.

Before adding final ingredients, temper medium to 48 C and to each batch of 100 ml add 1 ml of 0.1 % sterile (121 C for 20 min) aqueous solution of tetrazolium blue and 1 ml of 4 % sterile (121 C for 20 min) aqueous solution of sodium azide. Final pH 7.0 ± 0.2 @ 25 C.

Pour about 15 ml of the final prepared medium per plate. After solidification, stratify the plates with a thin layer of the same medium.

For enumeration of colonies, place a thin sheet of white paper (facial tissue) underneath a petri dish on the illuminated colony counter to enhance color contrast between colonies and the background.

B. Eosin Methylene Blue Agar, Levine:[10]

Note on preparation and use: Avoid overheating, as the agar is likely to become soft and unsuitable for streaking. When it is to be used as a streaking medium, melt agar in flowing steam or boiling water. Gently swirl contents of flask to distribute flocculant precipitate and pour a thick layer into petri plates. Set cover aside to leave about one-third of the plate exposed for escape of moisture vapor and to dry surface of agar after solidification. A dry surface promotes growth of distinct individual colonies and gives better type differentiation. Plates should be used within a week and can be stored inverted in the refrigerator. When stored, incubate non-inoculated plates as a check for contamination.

C. Lactose Broth:

Note on preparation: When fermentation tubes or other containers are prepared to examine 10-ml portions of samples, the lactose broth medium must be of such strength that addition of that volume of sample to the medium will not reduce the concentration of ingredients below that in the standard medium. When 10-ml portions are inoculated into 10 ml of medium, the medium must be formulated at double strength. When 10-ml portions are inoculated into 20 ml of medium, the medium must be formulated at 1.5 times the strength of the standard ingredients.

D. MF-Endo Broth:[6]

Note on use: For best results, medium should be prepared and used the

same day; however, it may be stored for not more than five days if kept in a tightly closed container, protected from bright light and refrigerated.

E. Rose Bengal Agar with chlortetracycline hydrochloride:[5]

Peptone	5	g
Glucose	10	g
Potassium dihydrogen phosphate (KH_2PO_4)	1	g
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.5	g
Agar	20	g
Distilled water to make	1	liter

Suspend ingredients in water and heat to boiling to dissolve completely. Dispense 100 ml of medium into screw-cap containers. Sterilize at 121 C for 15 minutes.

Prepare stock solution of 1% Rose Bengal in distilled water. Sterilize at 121 C for 15 minutes. Store at 4 C.

Prepare stock solution of chlortetracycline hydrochloride by dissolving 100 mg in 100 ml of distilled water. Store at 4 C for no more than one week.

To 100 ml of melted and cooled agar, add 0.2 ml of the Rose Bengal and 2 ml of the chlortetracycline hydrochloride stock solutions just before distributing into appropriate tubes or plates. Final pH 6.0 ± 0.2 @ 25 C.

F. Standard Methods Agar:

Formulation of Standard Methods Agar shall conform to that for Standard Methods Agar described in *Standard Methods for the Examination of Dairy Products*, 13 ed.[18] This is the same formulation described for Plate Count Agar, Dehydrated (Tryptone Glucose Yeast Agar) in *Standard Methods for the Examination of Water and Wastewater*, 14th ed.[19] It is also the same formulation as for Tryptone Glucose Yeast Agar of the *Association of Official Analytical Chemists*.[1] The formulation consists of the following ingredients and quantities per liter of medium:

Pancreatic digest of casein	5.0	g
Yeast extract	2.5	g
Glucose	1.0	g
Agar	15.0	g
Microbiologically suitable water to make	1	liter

Final pH should be 7.0 ± 0.2 @ 25 C after sterilization at 121 C for 15 minutes.

Ingredients used in formulation for Standards Methods Agar shall conform to the following specifications:

1. Pancreatic digest of casein—Shall be of USP quality as given in the U.S. Pharmacopoeia XIX, p 744.[20]

2. Yeast extract—Shall conform to specifications for yeast extract as given in the U.S. Pharmacopoeia XIX, p 758.[20]

3. Glucose—Shall conform to specifications for glucose as given in the U.S. Pharmacopoeia XIX, p 128 [21]; it shall be anhydrous.

4. Agar—Shall conform to the following specifications:

a. General characteristics: The dry material shall be in granule or flake form, cream-white to pale tan in color. An amount of 2.0 g should be evenly suspendable in 100 ml of cold distilled water without lumping. On application of heat, solution should be complete within 5 min at 100 C.

After autoclaving the mixture at 121 C for 15 min, a hot 2.0% solution may be clear or hazy, but should be without milkiness and free from extraneous matter or suspended particles which might be confused with bacterial colonies. After autoclaving and cooling to room temperature, the pH should be not less than 6.0.

b. Temperature of gelation and melting: A 1.5 % hot aqueous solution should, upon cooling, gel at not over 43 C, nor less than 33 C. After gelation, the solidified material should not melt at less than 70 C.

c. Viable spore count: The viable spore count should not exceed 50. Add 2 g of agar to 100 ml of sterile soybean casein digest broth USP, and autoclave the mixture at 121 C for 5 minutes. The mixture is rotated to obtain uniform distribution and, after cooling to 45 C is poured into three or four sterile petri plates. Incubate the plates for 48 hr at 32 to 35 C and count colonies. The total colony count, divided by 2, should not be more than 50.

G. Violet Red Bile Agar:

Note on preparation: Violet Red Bile Agar should not be autoclaved.[9] Instead, it should be boiled for 2 min, then used immediately as a plating medium after cooling to 45 C.

4.10 Physical Standards for Standard Methods Agar

Standard Methods Agar medium shall conform to the following requirements:

A. It shall be prepared from ingredients which meet specifications for ingredients of, and according to the formula for, Standard Methods Agar medium as described in 4.9(F) preceding.

B. It shall be a uniform free-flowing beige or light buff-colored powder.

C. It shall have a moisture content of less than 5.0 %. Moisture is to be determined using a moisture balance with a 125-W, 115- to 125-V infrared industrial reflector bulb; a Powerstat transformer set at 100 is to be in the line to prevent charring. The exposure shall be 15 minutes.

D. It shall be soluble in 2.35 % solution in distilled water upon boiling for 1 to 2 min to yield a slightly opalescent-to-clear solution without precipitate.

E. It shall have a final reaction of pH 7.0 ± 0.2 at 25 C after being autoclaved at 121 C for 15 minutes.

F. It shall not develop an objectionable precipitate when held at 50 to 56 C for 2 hr after autoclaving that could interfere with the Standard Plate Count.

4.11 Productivity Tests for Standard Methods Agar Medium

Productivity of each lot of medium shall be compared with the American Public Health Association (APHA) Reference Standard for Standard Methods Agar† with respect to a.) number of colonies and b.) size of the colonies. This primary standard must be used in all testing to determine acceptability of each lot; use of a secondary standard for official testing is not permissible.

This method was approved by the Coordinating Committee on Laboratory Methods (APHA) and was adopted by the APHA in 1965 as recommended.

The method compares series of replicate plates for the test medium with a series on the Reference Standard, using four milk composites. An acceptable test medium must yield counts within ± 10 % of those obtained on the Reference Standard. This protocol specifies that the testing laboratory achieve results with no more than 10 % coefficient of variation and requires 20 replicate plates per milk composite for each medium. If the laboratory can achieve a 5 % coefficient of variation, only 10 replicate plates per composite for each medium will be required [4.11(G)].

A. Preparation of reference and test media:

1. Prepare each medium according to the manufacturer's directions on the label of the container.

2. Dispense each medium in 15–ml amounts in tubes.

3. Sterilize according to the manufacturer's directions (generally at 121 C for 15 minutes.

B. Preparation of milk composites:

1. Prepare four composites of raw milk samples, using no less than five well-mixed individual milk samples for each composite. Mix the individual samples with a vortex mixer before each composite is made. Make composites as soon as possible (within 24 hr) after the samples are received in the laboratory. Pasteurized milk composites may be prepared and tested on different days from raw milk composites.

2. Do a preliminary count, if desired, to determine the most satisfactory dilution to use—that which will yield approximately 200 colonies per plate. If a preliminary count is not made, two dilutions will have to be plated for the comparison to be described.

3. If a preliminary count is made or if composites are to be held for testing, undiluted composites should be kept frozen and diluted to the appropriate dilution when ready for plating. Use composites within 72 hr after preparation.

C. Dilution of milk composites:

When ready to begin plating and testing of media, dilute each composite in 10–fold dilutions, using sterile phosphate-buffered water, which is prepared as described in 4.7(B).

†The Reference Standard is available from the American Public Health Association, 1015 Eighteenth Street, NW, Washington, DC 20036.

D. Plating of diluted milk composites:

1. Melt each medium in a steam bath and hold in a water bath at 44 to 46 C for no longer than 24 hr, provided that a precipitate does not form. Do not melt medium more than once.

2. Plate two consecutive dilutions, if preliminary counts have not been made to establish a countable dilution. Use dilutions of milk composites that will yield approximately 200 colonies per plate. The allowable plate count range is 100–300 colonies per plate, provided that no single plate count exceeds 300 and no average count is below 100.

3. Use a sterile 2-ml pipet (calibrated to deliver 2.0 ± 0.02 ml) for all plates for each dilution of each milk composite. Before sterilization, the pipet should be thoroughly cleaned so that liquid does not adhere to its wall.

E. Plating pattern:

Plating shall be done according to the following pattern:

1. Number plates according to the pipet–pour scheme given in Table 4:I.

Table 4:I. Two Milk Composite Schemes

COMPOSITE I		COMPOSITE II	
Standard (Pour First)	Test (Pour Second)	Standard (Pour Second)	Test (Pour First)
S–1	T–1	S–1	T–1
S–2	T–2	S–2	T–2
S–3	T–3	S–3	T–3
S–4	T–4	S–4	T–4
S–5	T–5	S–5	T–5
(Pour Second)	(Pour First)	(Pour First)	(Pour Second)
S–6	T–6	S–6	T–6
S–7	T–7	S–7	T–7
S–8	T–8	S–8	T–8
S–9	T–9	S–9	T–9
S–10	T–10	S–10	T–10
(Pour First)	(Pour Second)	(Pour Second)	(Pour First)
S–11	T–11	S–11	T–11
S–12	T–12	S–12	T–12
S–13	T–13	S–13	T–13
S–14	T–14	S–14	T–14
S–15	T–15	S–15	T–15
(Pour Second)	(Pour First)	(Pour First)	(Pour Second)
S–16	T–16	S–16	T–16
S–17	T–17	S–17	T–17
S–18	T–18	S–18	T–18
S–19	T–19	S–19	T–19
S–20	T–20	S–20	T–20

2. Rinse pipet thoroughly with diluted milk composite before pipetting the sample.

3. Place 2 ml of the diluted milk composite in each plate, S–1 through S–5 and T–1 through T–5, *allowing milk to flow freely from the pipet. Drain for 5 seconds. Touch pipet tip briefly to a dry portion of the plate*. Pour one tube of Reference Standard medium (15 ml) into each plate S–1 through S–5, rotating each plate, as poured, to achieve adequate dispersal of inoculum. Repeat with the test medium in plates T–1 through T–5.

4. Pipet the diluted milk to plates T–6 through T–10, then to S–6 through S–10. Pour appropriate medium into each plate in accordance with Table 4:I. If 20 plates are required for each medium, continue this procedure with plates S–11 through S–15, then with T–11 through T–15, followed by plates T–16 through T–20 and S–16 through S–20.

5. Reverse order for the second composite. Repeat for the other type of milk composite.

6. Invert all plates; incubate at 32 C for 48 hours.

F. Enumeration and colony size:

Use a colony counter for enumeration. Productivity with respect to size shall be considered comparable if the colonies on the test medium are generally similar in size to those obtained on the reference medium, using the required number of samples as specified.

G. Statistical analysis and interpretation of results:

The method of comparison just described requires certain limits on coefficients of variation for the media involved. To calculate the coefficient of variation for counts from each medium, let

X = plate count obtained

and N = number of replicate plates.

Calculate: the arithmetic mean (or average) plate count for reference and test media:

$$\overline{X} = \frac{\Sigma X}{N}.$$

and the sample standard deviation for reference and test media:

$$s = \sqrt{\frac{\Sigma X^2 - \frac{(\Sigma X)^2}{N}}{N - 1}}$$

Then calculate the coefficient of variation by:

$$C.V. = \left(\frac{s}{\overline{X}}\right)100.$$

(This is usually expressed as a percentage.)

When 20 plates are used for each medium, the coefficient of variation cannot exceed 10%. If this value is exceeded for either medium, repeat replicates on *both* media until satisfactory precision is obtained. When 10 plates

are used for each medium, the coefficient of variation cannot exceed 5%. If this value is exceeded for either medium, repeat replicates on *both* media until satisfactory precision is obtained.

Once satisfactory precision has been achieved, the average counts on the two media are compared by calculating the corresponding Student's t–value. This is done as follows:

let $\overline{X}_R$ = average plate count for the reference medium

$\overline{X}_T$ = average plate count for the test medium

N = number of plates counted for each type of medium‡

s_R^2 = variance for the reference medium

and

s_T^2 = variance for the test medium,

then calculate Student's t by

$$t = \frac{|\overline{X}_R - \overline{X}_T|}{\sqrt{\frac{s_R^2 + s_T^2}{N}}}$$

For each comparison, the t–value should be less than 2.70 when N = 20, or less than 2.88 when N = 10. (A 1% level of significance is used for this statistical test, corresponding values at the 5% level are 2.02 and 2.55, respectively.) If the calculated t–value for any milk comparison exceeds the above values, the test medium should be rejected.

H. Statistical considerations for the number of replicates required:

The number of replicates required to detect a prescribed difference between average counts of test and reference media depends on the following factors:[14]

d = the difference to be detected between average counts of the test medium and the reference medium. This difference is of practical importance

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‡If different numbers of plates are used for some reason, proceed as follows:

Let N_R = number of plates counted for the reference medium

N_T = number of plates counted for the test medium, then

$$t = \frac{|\overline{X}_R - \overline{X}_T|}{\sqrt{\frac{(N_R + N_T)}{N_R N_T}\left(\frac{s_R^2(N_R - 1) + s_T^2(N_T - 1)}{N_R + N_T - 2}\right)}}.$$

This is distributed as Student's t with $N_R + N_T - 2$ degrees of freedom. The appropriate significance levels can be found in a table of t–values.

when it is at least 10% of the average count of the reference medium. (*i.e.*, $d = .10\ \overline{X}_R$)

σ = standard deviation, a measure of the variability in counts. On the basis of experimental tests, it may be assumed that the standard deviation is the same for counts obtained on the reference medium and on the test medium. The magnitude of the standard deviation was found to be around 10% of the average count. By requiring each comparative series of plates to exhibit no greater than 10% coefficient of variation, an estimate may be established for the standard deviation as 10% of the average count for the reference medium (*i.e.*, $\sigma = .10\ \overline{X}_R$). For more precise results, if each comparative series of plates is required to exhibit no greater than 5% coefficient of variation, an estimate may be established for the standard deviation as 5% of the average count for the reference medium. (*i.e.*, $\sigma = .05\ \overline{X}_R$).

a = level of significance, the probability of concluding that there is a difference between average counts obtained on the reference medium and on the test medium when, in fact, there is none. This probability should be small. Accordingly, a 1% ($\alpha = .01$) level of significance has been used in the following table.

β = the probability that there is no difference between the average counts of the test medium and of the reference medium when, in fact, a difference does exist. This probability should also be small. Accordingly, values of 0.05 and 0.01 were employed for β in the following table.

To determine the number of replicates required by the above criteria, the "standardized difference" D, where $D = \frac{d}{\sigma}$, is computed. This ratio depends on d (the magnitude of the difference to be detected) and on σ (the standard deviation). Three situations are presented below relating d and σ:

1. Assume that the testing laboratories can achieve results with coefficients of variation of 10%, and that a 5% difference is to be detected between average counts obtained on the test medium and the reference standard, then

$$D = \frac{d}{\sigma} = \frac{.05\ \overline{X}_R}{.10\ \overline{X}_R} = \frac{.05}{.10} = .50.$$

2. Assume that the testing laboratories can achieve results with coefficients of variation of 5% and that a 5% difference is to be detected between the test medium and the reference standard, then

$$D = \frac{d}{\sigma} = \frac{.05\ \overline{X}_R}{.05\ \overline{X}_R} = 1.00.$$

Similar calculations can be used for a 10% coefficient of variation and a 10% difference, and

3. Assume that the testing laboratories can achieve results with coefficients of variation of 5% and that a 10% difference is to be detected, then

$$D = \frac{d}{\sigma} = \frac{.10\,\overline{X}_R}{.05\,\overline{X}_R} = 2.00.$$

The values of D and the number of replicates required in each situation described above are presented in Table 4:II, assuming that $\alpha = 0.01$.

Table 4:II. Number of Replicate Plates for Each Medium to be Tested

Case	D	# plates $\beta = .05$	# plates $\beta = 0.1$
(1)	0.5	74	99
(2)	1.0	20	27
(3)	2.0	7	9

4.12 References

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